Published in "Antimicrobial Agents and Chemotherapy doi: 10.1128/AAC.00127-17, 2017"
which should be cited to refer to this work.

In-vitro study of IS*Apl1*-mediated mobilization of the colistin resistance gene *mcr-1*

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Running title ; Functional transposition of *mcr-1*

Keywords ; polymyxin, plasmid, *mcr-1*, IS*Apl1*, transposition, composite transposon

The plasmid-mediated *mcr-1* gene encodes a phosphoethanolamine transferase conferring resistance to polymyxins. The *mcr-1* gene is associated with insertion sequence IS*Apl1* (IS*30* family). In-vitro mobilization assays demonstrated the functionality of the composite transposon structure IS*Apl1*-*mcr-1*-IS*Apl1*. Transposition generated a 2-bp duplication and occurred in AT-rich DNA regions. This is the first report demonstrating the mobility of the *mcr-1* gene by transposition.

Since its discovery by the end of 2015 (1), the occurrence of the plasmid-mediated colistin resistance gene *mcr-1* has been reported worldwide. This gene has now been reported from human, environmental, and animal samples (2), and also from retail food (3), in a variety of enterobacterial species even though mostly in *Escherichia coli*. Retrospective studies reported MCR-1-producing colistin-resistant isolates as early as in the late 1980s (4), but several studies suggest that spread of the *mcr-1* gene is on a rising trend (5).

Various plasmids may carry the *mcr-1* gene including those belonging to the incompatibility groups IncX4, IncI2, InHI2, IncF, IncY and IncP (6-10). This gene is often identified in association with the insertion sequence IS*Apl1* that may play a major role in its mobilization (11-13).

IS*Apl1* belongs to the IS*30* family and was first identified in *Actinobacillus pleuropneumoniae* (14), a Gram-negative rod of the *Pasteurellaceae* family being a causative agent of porcine necrotic pleuropneumonia. It is a 1,070-bp long mobile element possessing a 924-bp ORF encoding a 307 amino-acid transposase containing a DDE domain containing the carboxylate residues believed to be responsible for coordinating metal ions needed for catalysis. It is flanked by two imperfect 27 bp inverted repeats (IRs) exhibiting 6 base-pair mismatches. In a recent study (11), an intermediate circular form of IS*Apl1* associated with

mcr-1 was detected, suggesting that IS*Apl1* might be involved in the mobilization of this resistance gene. Moreover, a ca. 790-bp open reading frame has been identified downstream of the *mcr-1* gene in most of the MCR-1 producers. This sequence is believed not playing any role in colistin resistance (15) nevertheless its putative role in the mobilization of the *mcr-1* gene remains to be determined. Recent works (6, 16) showed that *mcr*-1 is part of a 2,600-bp cassette containing promoter sequences for *mcr*-1 expression, and bracketed in most cases by two direct copies of IS*Apl1*, suggesting that it may constitute a composite transposon element. Therefore, the aim of our study was to determine experimentally whether IS*Apl1* could actually mobilize the *mcr-1* gene.

Since preliminary experiments showed that cloning the *mcr-1* gene in regular recombinant vectors is difficult, likely due to a toxic effect of MCR-1 once overproduced in *E. coli* (data not shown), a truncated form of *mcr-1* was created by inserting the $bla_{\text{TEM-1}}$ gene into the coding sequence of *mcr-1*, as shown in Figure 1. Then, two structures were analyzed, being the entire composite transposon bracketed by two copies of IS*Apl1* encompassing the *mcr-1*:$bla_{\text{TEM-1}}$ gene, and the same structure deleted from the right-hand copy of IS*Apl1*. Those two different genetic structures, namely IS*Apal1-mcr-1*:$bla_{\text{TEM-1}}$-*orf*-IS*Apl1* and IS*Apl1-mcr-1*:$bla_{\text{TEM-1}}$-*orf* , respectively, were obtained by PCR and then ligated and cloned

into plasmid pNK1 (p15A-pTOPO-ΔlacP-Kan^{R}) giving rise to recombinant plasmids pNK31 and pNK45, respectively. Those plasmids were transformed into *E. coli* TOP10 (InvitroGen®, Thermo Fisher Scientific, Ecublens, Switzerland) and selected onto Luria-Bertani (LB) agar plates supplemented with 100 µg/ml of ampicillin and 25 µg/ml of kanamycin. Plasmids pNK31 and pNK45 were then transformed into the *E. coli* strain RZ211 containing the transfer-proficient pOX38 F plasmid carrying a gentamicin resistance gene (17). Plasmid pOX38 is a self-conjugative and IS-free plasmid encoding resistance to gentamicin that serves as a target for transposition events that may be searched searched after 24 h of growth as described (18). By conjugating the pOX38 plasmid into another *E. coli* recipient strain using gentamicin as selective marker, it is therefore possible to isolate and identify putative transposition events.

Clones were selected onto LB agar plates supplemented with ampicillin (100 µg/ml), kanamycin (25 µg/ml) and gentamicin (10 µg/ml). *E. coli* RZ211 harboring recombinant plasmids pNK31 or pNK45 were used as donors for conjugation experiments with the azide-resistant *E. coli* strain J53. Briefly, the donor and recipient strains were separately cultured overnight and then subcultured for 5 h in order to reach the exponential phase. Mating-out assay were performed on solid media using filters with a 1:10 donor:recipient ratio. After 5 h

of incubation, filters were resuspended in NaCl 0.85% and bacterial mixtures were plated onto agar plates supplemented with gentamicin (10 µg/ml) and sodium azide (100 µg/ml) or onto agar plates supplemented with gentamicin, sodium azide and ampicillin (100 µg/ml). All $Gen^{R}Azide^{R}Amp^{R}$ colonies were screened for kanamycin susceptibility to exclude the spontaneous *E. coli* RZ211 azide-resistant mutants, or possible co-integration events that might not correspond to transposition events. The transposition frequency was obtained by dividing the number of $Gen^{R}Azide^{R}Amp^{R}Kan^{S}$ colonies by the number of $Gen^{R}Azide^{R}$ transconjugants. In total, we selected randomly 100 $Gen^{R}Azide^{R}Amp^{R}Kan^{S}$ transposants recovered from the conjugation experiment using RZ211-pNK31 as donor and identified seven distinct transposition events (Figure). No transposant was found with the strain RZ211-pNK45 used as donor (only a single copy of IS*Apl1*). The transposition frequency determined in *E. coli* J53 with pNK-31 as donor plasmid was estimated to be at 2.2×10^{-8} which is relatively low. The insertion sites of the IS*Apl1*-*mcr-1*:bla_{TEM-1}-*orf*-IS*Apl1* cassette were determined by using an inverse PCR strategy. Briefly, DNA from the transposants was extracted using the GenElute™ Bacterial genomic kit (Sigma Aldrich) and was digested by the PstI restriction enzyme (InVitrogen®). Digested fragments were self-circularized by ligation and used as templates for reverse PCR using outward primers as listed in Table 1 and

indicated on Figure. Since two PstI restriction sites were located into the bla_{TEM-1} and *mcr-1* genes, respectively, two PCR amplifications per transposant were performed. The first PCR amplification was performed using primers IS*Apl1*-SP3 and TEM-Fw and a second PCR was performed using primers mcr-south-Rv and IS*Apl1*-3'-Fw in order to characterize the 5' and 3' genetic contexts of the insertions, respectively. Sequencing of the corresponding amplicons revealed that transposition events occurred in seven different sites namely Ins-1 to Ins-7 (Figure 2A), the whole mobilized transposon being always 5,699-bp in size. Each transposition event generated 2-bp direct repeats at the insertion site (Figure 2B). High AT-rich DNA sequences were identified on the two flanking regions of all insertion sites (Figure 2B). Our data are in accordance with previous studies showing that IS*Apl1* like other IS*30*-like elements targets preferentially AT-rich sequences (14). Noteworthy, in-silico analysis showed that in most of the sequenced plasmids, the *mcr-1* gene is flanked by AT-rich regions.

Our results support therefore the hypothesis made by Snesrud et al. suggesting that the mobilization of *mcr-1* is mediated by a composite transposon (12). The fact that the *mcr-1* gene was associated with only a single copy of IS*Apl1* at its 5' extremity in some studies might be explained by the characteristic of IS*30* family members to excise one copy of the IS element by transposition or by illegitimate recombinations events after transposition of the

original composite transposon, in order to stabilize the genetic structure once integrated (19, 20). This hypothesis agrees with the lack of transposition event observed using the pNK45 construct.

Here, we demonstrate the effective mobilization of the *mcr-1* gene located into a composite transposon named Tn*6330.2* based on in-silico comparison with Tn*6330* (11). This work confirms previous hypotheses (12) considering that the *mcr-1* gene had been initially mobilized by two copies of IS*Apl1* from an unknown progenitor, targeting broad-host range plasmid(s) that subsequently transferred this resistance gene into Enterobacteriaceae. Interestingly, our very recent work showed that *Moraxella* species are natural sources of *mcr*-like genes, and may harbor IS*Apl1* elements (21). Therefore, mobilization of the *mcr-1* gene might have occurred into a *Moraxella* species (still to be precisely identified) an IS*Apl1*-mediated transposition process. Further studies are being conducted to reproduce mobilization of *mcr*-like genes from such bacterial sources by IS*Apl1*-mediated transposition.

ACKNOWLEDGMENTS

This work has been funded by the University of Fribourg, by grants from the ANIWHA ERA-NET project, Switzerland, by the OFSP, Bern, Switzerland (grant n°16009294), and by the Novartis Foundation for medical-biological Research.

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Legends

Fig. 1. Schematic map of the different constructs performed for the transposition study. (A) corresponds to the original transposon IS*Apl1-mcr-1-orf*-IS*Apl1* identified in clinical isolates; (B) shows the different fragments generated by PCR (with corresponding restriction sites indicated) used as templates for ligation and subsequent genesis of (C) IS*Apl1-mcr-1*:*bla*$_{TEM-1}$-*orf*-IS*Apl1* or (D) IS*Apl1-mcr-1*:*bla*$_{TEM-1}$-*orf* genetic structures. Locations of primers used for the inverse PCR strategy (as listed in Table 1) are indicated by small half arrows. Restriction sites of endonucleases used for cloning are indicated (NheI, SacI, EcoRI, and BamHI).

Fig. 2. Target sites of the IS*Apl1-mcr-1*:*bla*$_{TEM-1}$-*orf*-IS*Apl1* composite transposon. (A) Map positions of IS*Apl1-mcr-1*:*bla*$_{TEM-1}$-*orf*-IS*Apl1* composite transposon in plasmid pOX38-Gen. Insertions of the tagged insertion sequence (Ins-1 to -4) are indicated by a vertical arrow. (B) Nucleotide sequence alignment of the three IS*Apl1-mcr-1*:*bla*$_{TEM-1}$-*orf*-IS*Apl1* transposon events identified into pOX38-Gen. Nucleotide sequences of the end regions of transposon are boxed. Boldfaced letters indicate target site sequences duplicated upon transposition. Orientation of the insertion sequences are indicated by (+) and (-).

Table 1. Sequence of primers used in this study

Primer	Sequence(5′ → 3′)	Position in figure 1
ISAplSP3	CAGGCTGCTCTAATTTGCGC	1
IS*Apl1*-3′-Fw	AGACATCAATCAGTGGAGCG	4
mcr-south-Rv	GATAGACACCGTTCTCACCC	3
Nhe-ISApl1	GATGATGCTAGCGCTGAATTTACAATCCAAGT	
SacI-Δmcr-1	GATGATGAGCTCGTAGGGCATTTTGGAGCATG	
Sac-I-TEM-1	GATGATGAGCTCGTATCCGCTCATGAGACAATA	2
EcoRI-TEM-1	GATGATGAATTCTCTAAAGTATATATGAGTAAACTTGGTCTG	
EcoRI-Δmcr-1	GATGATGAATTCCCGAGACCAAGGATCTATTA	
BamHI-Cass	GATGATGGATCCGTTATTTCTGTTTGGGGTTG	
BamHI-ISApl1	GATGATGGATCCCATTGCGCAATCCCATACTG	

Table 2. Strains used in this study

Strain	Species	Features
Af31	*E. coli*	MCR-1 producer clinical isolate carrying two copies of IS*Apl1*
Af45	*E. coli*	MCR-1 producer clinical isolate carrying only one copy of IS*Apl1*
RZ211	*E. coli*	Isolate carrying the pOX38-Gen plasmid
J53	*E. coli*	Azide-resistant isolate used for mating-out experiments

Figure 1

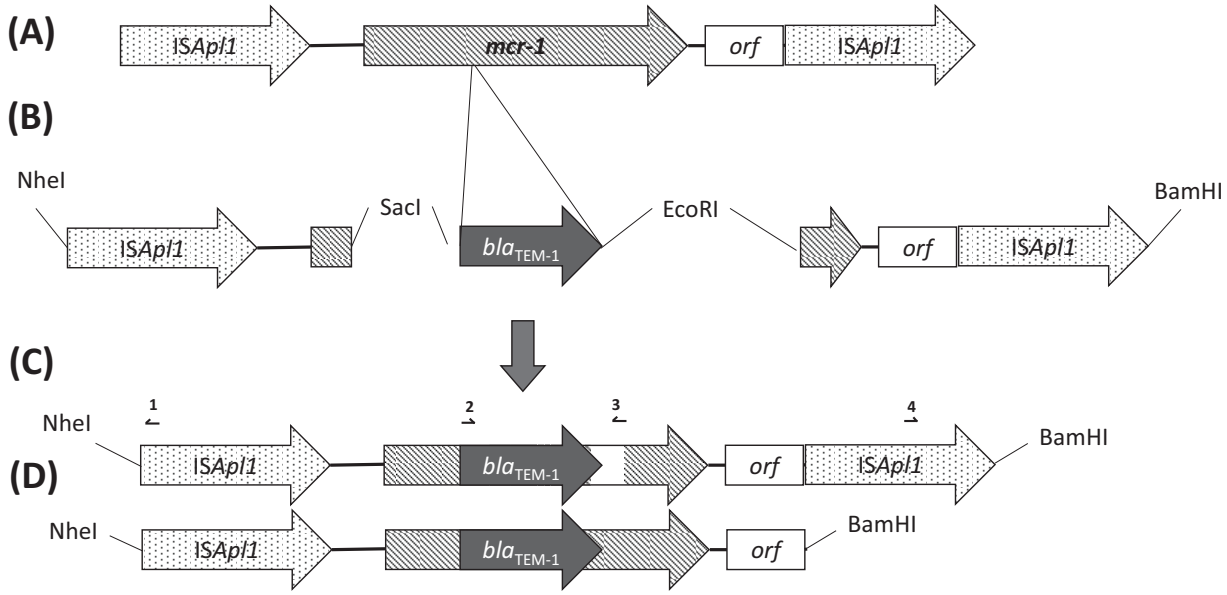
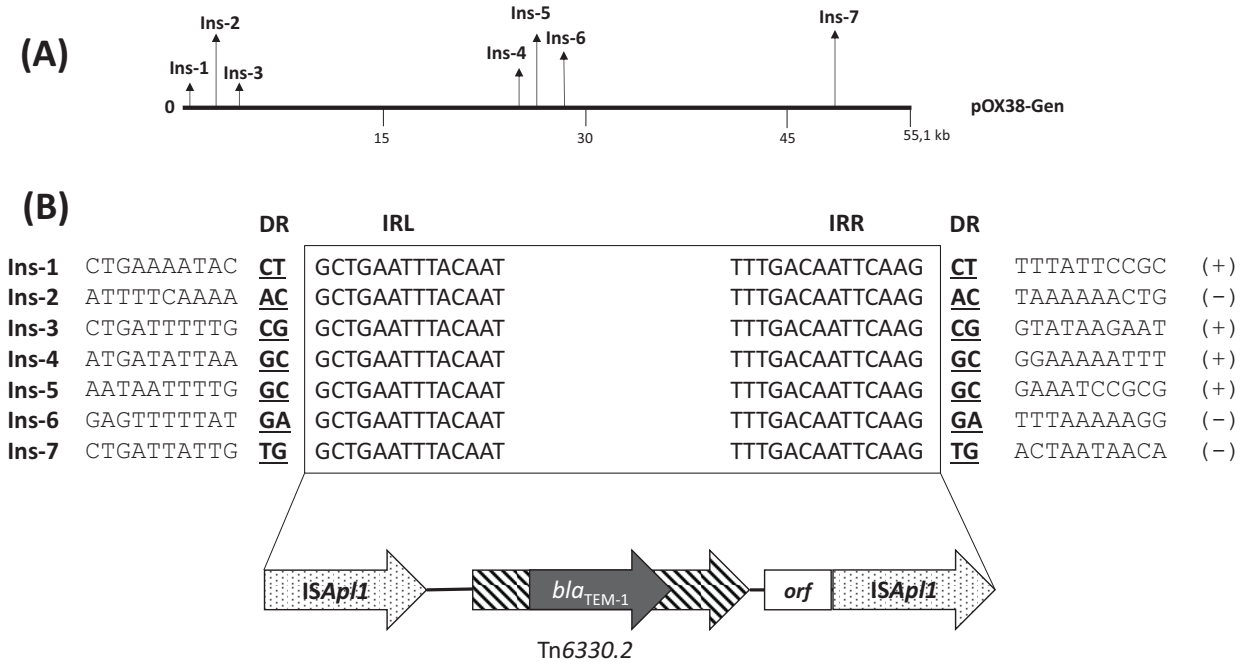

(A)
ISApl1
mcr-1
orf
ISApl1
(B)
NheI
SacI
EcoRI
BamHI
ISApl1
bla TEM-1
orf
ISApl1
(C)
1
2
3
4
NheI
BamHI
(D)
ISApl1
bla TEM-1
orf
ISApl1
NheI
BamHI
ISApl1
bla TEM-1
orf

Figure 2


(A)
Ins-1
Ins-2
Ins-3
Ins-4
Ins-5
Ins-6
Ins-7
0
15
30
45
55,1 kb
pOX38-Gen
(B)
DR
IRL
IRR
DR
Ins-1 CTGAAAATAC CT GCTGAATTTACAAT TTTGACAATTCAAG CT TTTATTCCGC (+)
Ins-2 ATTTTCAAAA AC GCTGAATTTACAAT TTTGACAATTCAAG AC TAAAAAACTG (−)
Ins-3 CTGATTTTTG CG GCTGAATTTACAAT TTTGACAATTCAAG CG GTATAAGAAT (+)
Ins-4 ATGATATTAA GC GCTGAATTTACAAT TTTGACAATTCAAG GC GGAAAAATTT (+)
Ins-5 AATAATTTTG GC GCTGAATTTACAAT TTTGACAATTCAAG GC GAAATCCGCG (+)
Ins-6 GAGTTTTTAT GA GCTGAATTTACAAT TTTGACAATTCAAG GA TTTAAAAAGG (−)
Ins-7 CTGATTATTG TG GCTGAATTTACAAT TTTGACAATTCAAG TG ACTAATAACA (−)
ISApl1
bla TEM-1
orf
ISApl1
Tn6330.2